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(54) Title: COMPOSITIONS AND PROCESSES USING siRNA, AMPHIPATHIC COMPOUNDS AND POLYCATIONS

(57) Abstract: Described is a deliverable composition with low toxicity comprising an amphipathic compound, a polycation, and a siRNA. The composition may be used in the process of delivering a siRNA to an animal cell or more particularly, a mammal cell.

Compositions and Processes Using siRNA, Amphipathic Compounds and Polycations

Field of the Invention

- 5 The field of the present invention is a composition comprising siRNA, amphipathic compounds and polycations and the use of such compositions for delivering the siRNA to an animal cell.

Background

- 10 RNA interference (RNAi) is a phenomenon wherein double-stranded RNA, when present in a cell, inhibits expression of a gene that has an identical or nearly identical sequence. Inhibition is caused by degradation of the messenger RNA (mRNA) transcribed from the target gene ¹. The double-stranded RNA responsible for inducing RNAi is termed interfering RNA. The mechanism and cellular machinery through which dsRNA mediates RNAi has been
- 15 investigated using both genetic and biochemical approaches. Biochemical analyses suggest that dsRNA introduced into the cytoplasm of a cell is first processed into RNA fragments 21-25 nucleotides long ^{2,3,4,5,6}. It has been shown in *in vitro* studies that these dsRNAs, termed small interfering RNAs (siRNA) are generated at least in part by the RNase III-like enzyme Dicer ⁷. These siRNAs likely act as guides for mRNA cleavage, as the target mRNA is
- 20 cleaved at a position in the center of the region covered by a particular siRNA ⁸. Biochemical evidence suggests that the siRNA is part of a multicomponent nuclease complex termed the RNA-induced silencing complex (RISC) ². One of the proteins of this complex, Argonaute2, has been identified as a product of the argonaute gene family ⁹. This gene family, which also contains the *C. elegans* homolog rde-1 and related genes, the *N. crassa* homolog qde-2, and
- 25 the *Arabidopsis* homolog arg-1, has been shown to be required for RNAi through genetic studies ^{10,11,12}. Genetic screens in *C. elegans* have also identified the mut-7 gene as essential for RNAi. This gene bears resemblance to RNase D, suggesting that its gene product acts in the mRNA degradation step of the reaction ¹³.
- 30 Although the use of easily manipulated model systems such as *C. elegans* and *D. melanogaster* in gene function studies can yield clues concerning possible new drug targets in mammals, a more direct approach would be to study gene function in mammalian model systems. It has previously been demonstrated that dsRNA can be used to induce RNAi and inhibit target gene expression in mouse oocytes and early embryos ^{14,15}. However, data

obtained in a number of other studies have indicated that the use of dsRNA to induce RNAi in cultured mammalian cells or post-embryonic tissue may not be effective as a sequence-specific method of gene silencing^{16,17}. This discrepancy may be due in large part to the well-documented dsRNA-mediated induction of interferon synthesis, a response pathway not
5 present in oocytes and early embryos. Activation of dsRNA dependent enzymes leads to non-sequence specific effects on cellular physiology and gene expression^{18,19,20,21}. A major component of the interferon response is the interferon-induced dsRNA-dependent protein kinase, protein kinase R (PKR), which phosphorylates and inactivates the elongation factor eIF2a. In addition, interferons induce the synthesis of dsRNA dependent 2-5(A) synthetases,
10 which synthesize 2'-5' polyadenylic acid leading to the activation of the non-sequence specific RNase L²².

The PKR pathway however, is not activated by dsRNA of less than 30 base pairs in length and full activation requires dsRNAs 80 base pairs in length^{19,20}. This fact suggested that if
15 siRNAs are used to initiate RNAi instead of longer dsRNAs, it would be possible to circumvent at least part of the interferon response. Data obtained from studies in which siRNA 21-25 base pairs in length was delivered to mammalian cells in culture indicated that sequence-specific inhibition through RNAi is indeed effective^{23,24}. In these studies, gene-specific inhibition was observed in a variety of both immortalized and primary cell lines. The
20 degree of inhibition varied between 80-96% using siRNA targeted against a reporter gene expressed from transiently transfected plasmids containing strong enhancers. Expression of a control reporter gene of unrelated sequence was unaffected by the siRNA, and no inhibition was observed using siRNAs against unrelated sequences. Expression of endogenous genes could also be inhibited to levels below detection by siRNA. These data demonstrate the
25 specificity and effectiveness of siRNA-mediated RNAi in cultured mammalian cell lines and suggest that the interferon response is not activated by siRNAs of this length. These results suggest that RNAi can indeed be used to effectively inhibit gene expression in mammalian cells.

30 The ability to specifically inhibit expression of a target gene by RNAi has obvious benefits. For example, many diseases arise from the abnormal expression of a particular gene or group of genes. RNAi could be used to inhibit the expression of the deleterious gene and therefore alleviate symptoms of a disease or even provide a cure. For example, genes contributing to a cancerous state or to viral replication could be inhibited. In addition, mutant genes causing

dominant genetic diseases such as myotonic dystrophy could be inhibited. Inflammatory diseases such as arthritis could also be treated by inhibiting such genes as cyclooxygenase or cytokines. Examples of targeted organs would include the liver, pancreas, spleen, skin, brain, prostate, heart etc. In addition, RNAi could be used to generate animals that mimic true genetic "knockout" animals to study gene function.

Drug discovery could also be facilitated by siRNA technology. The siRNA approach for target validation will provide a quicker and less expensive approach to screen potential drug targets. Information for drug targeting will be gained not only by inhibiting a potential drug target but also by determining whether an inhibited protein, and therefore the pathway, has significant phenotypic effects. For example, inhibition of LDL receptor expression should raise plasma LDL levels and, therefore, suggest that up-regulation of the receptor would be of therapeutic benefit. Expression arrays can be used to determine the responsive effect of inhibition on the expression of genes other than the targeted gene or pathway²⁵. It will place the gene product within functional pathways and networks (interacting pathways).

The efficient delivery of biologically active compounds to the intracellular space of cells has been accomplished by the use of a wide variety of vesicles. One particular type of vesicle, liposomes, is one of the most developed types of vesicles for drug delivery. Liposomes, which have been under development since the 1970's, are microscopic vesicles that comprise amphipathic molecules which contain both hydrophobic and hydrophilic regions. Liposomes can be formed from one type of amphipathic molecule or several different amphipathic molecules. Several methods have been developed to complex biologically active compounds with liposomes. In particular, polynucleotides complexed with liposomes have been delivered to mammalian cells. After the description of DOTMA (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride)²⁶, a number of cationic lipids have been synthesized for this purpose. Essentially all the cationic lipids are amphipathic compounds that contain a hydrophobic domain, a spacer, and positively-charged amine(s). The cationic lipids are sometimes mixed with a fusogenic lipid such as DOPE (dioleoyl phosphatidyl ethanolamine) to form liposomes. The cationic liposomes are then mixed with plasmid DNA and the binary complex of the DNA and liposomes are applied to cells in a tissue culture dish or injected *in vivo*. The ease of mixing the plasmid DNA with the cationic liposome formulation, the ability of the cationic lipids to complex with DNA and the relative high levels of transfection efficiency has led to increasing use of these formulations. However, these cationic lipid

formulations have a common deficiency in that they are typically toxic to the cells in culture and *in vivo*.²⁷²⁸ More recently lipids have been used in association with other DNA-binding compounds to facilitate transfection of cells. The present invention provides new amphipathic compounds, and methods of preparation thereof, to be used to prepare novel complexes of
5 biologically active polyions for delivery to animal cells *in vitro* and *in vivo*. The complexes facilitate high efficiency transfer of the polyion from outside the cell to the inside a cell with low toxicity.

The present invention describes transfection reagents and methods to deliver siRNA to
10 animal cells *in vitro* and *in vivo* with high efficiency and low toxicity. We demonstrate that our method effectively delivers siRNA to animal cells for the purpose of RNA interference.

Summary

The present invention provides siRNA transfer into animal cells using a ternary complex
15 comprising siRNA, an amphipathic compound, and a polycation. Novel amphipathic compounds and methods of preparation thereof, are described. In a preferred embodiment, compositions comprising siRNAs, amphipathic compounds and polycations, and processes using such compositions to deliver a siRNA to an animal cell *in vivo* or *in vitro* for the purposes of inhibiting expression of a gene in the cell are described.

20 In a preferred embodiment, compositions and compounds are described that facilitate delivery of siRNA to an animal cell *in vitro* and *in vivo*. The siRNA comprises a double-stranded structure having a nucleotide sequence substantially identical to an expressed target gene within the cell. Further, the use of a polycation and a novel amphipathic compound
25 together significantly increased siRNA transfer efficiency. The siRNA then inhibits expression of a selected target gene.

In a preferred embodiment, the polycation is a polymer such as poly-L-lysine, polyethylenimine(PEI), polysilazane, polydihydroimidazolium, polyallylamine and the
30 like. A preferred cationic polymer is ethoxylated polyethylenimine (ePEI).

In a preferred embodiment the polycation is a DNA-binding protein. A preferred DNA-binding protein is a histone such as H1, H2A, or H2B. The histone can be from a natural source such as calf thymus or can be recombinant protein produced in bacteria. DNA-binding

proteins such as histone have several advantages over polycationic compounds such as polylysine. Human H1 histone protein is not immunogenic and does not induce anaphylaxis. Polylysine induces anaphylactic shock and is very immunogenic. In a preferred embodiment, the DNA-binding protein is linked to a nuclear localization signal such as a recombinant

5 histone produced in bacteria containing both the SV40 large T antigen nuclear localization signal and the C-terminal domain of human histone H1, (NLS-H1).

In a preferred embodiment, polyethylenimine or a similar polymer is used as the polycation and a compound of structure #1 is used as the amphipathic compound. In another preferred

10 embodiment, histone H1 protein is used as the polycation and a compound of structure #1 is used as the amphipathic compound. The siRNA can be used to study the target gene's effect on the cell or to affect a therapeutic change in the cell.

In a preferred embodiment, the cell can be an animal cell that is maintained in tissue culture

15 such as cell lines that are immortalized or transformed. These include a number of cell lines that can be obtained from American Type Culture Collection (Bethesda) such as, but not limited to: 3T3 (mouse fibroblast) cells, Rat1 (rat fibroblast) cells, CHO (Chinese hamster ovary) cells, CV-1 (monkey kidney) cells, COS (monkey kidney) cells, 293 (human embryonic kidney) cells, HeLa (human cervical carcinoma) cells, HepG2 (human

20 hepatocytes) cells, Sf9 (insect ovarian epithelial) cells and the like.

In another preferred embodiment, the cell can be a primary or secondary cell which means that the cell has been maintained in culture for a relatively short time after being obtained from an animal. These include, but are not limited to, primary liver cells and primary muscle

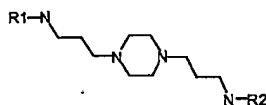
25 cells and the like. The cells within the tissue are separated by mincing and digestion with enzymes such as trypsin or collagenases which destroy the extracellular matrix. Tissues consist of several different cell types and purification methods such as gradient centrifugation or antibody sorting can be used to obtain purified amounts of the preferred cell type. For example, primary myoblasts are separated from contaminating fibroblasts using Percoll

30 (Sigma) gradient centrifugation.

In another preferred embodiment, the cell can be an animal cell that is within the tissue *in situ* or *in vivo* meaning that the cell has not been removed from the tissue or the animal.

In a preferred embodiment a process is describes for delivering an siRNA into an animal cell for the purposes of inhibiting expression of a gene (called RNA interference) comprising forming a complex comprising an amphipathic compound, an effective amount of a polycation and an siRNA, in solution, and associating the cell with the ternary comple. A
 5 preferred amphipathic compound is a compound of structure #1. A preferred polycation is ethoxylated PEI. Another preferred polycation is a histone.

A variety of amphipathic compounds can be used in conjunction with a polycation to mediate the transfer of the siRNA into the cell. In a preferred embodiment the amphipathic compound
 10 is cationic. The cationic amphipathic compound can be a non-natural polyamine wherein one or more of the amines is bound to at least one hydrophobic moiety wherein the hydrophobic moiety comprises a C6-C24 alkane, C6-C24 alkene, sterol, steroid, lipid, fatty acid or hydrophobic hormone. The amphipathic compounds may or may not form liposomes. In a preferred embodiment, several novel amphipathic cationic compounds are described. These
 15 compounds have the general structure comprising:



structure #1

wherein R₁ and R₂ come from the group consisting of C6-C24 alkane, C6-C24 alkene, sterol, steroid, lipid, fatty acid or hydrophobic hormone or other similar hydrophobic group. R₁ and
 20 R₂ may be identical or they may be different.

In contrast to the use of previously described cationic liposomes for gene transfer, most of the novel amphipathic cationic compounds described above do not efficiently mediate the transfer of genes into cells when used alone. However, the use of polycations with these
 25 novel amphipathic cationic compounds enables the efficient gene transfer into a variety of animal cells with minimal cellular toxicity. The combination of polycation and amphipathic compounds enhances the efficiency of siRNA delivery.

In a preferred embodiment, the present invention provides a process for delivering a siRNA
 30 to an animal cell comprising; preparing a ternary complex comprising mixing a compound of structure #1 with a siRNA and a polycation in a solution, associating the complex with an animal cell, and delivering the siRNA to the interior of the cell. The siRNA then inhibits

expression of a gene in the cell. The amphipathic compound may be mixed with the polycation prior to addition of the siRNA, at the same time as the siRNA, or after the siRNA.

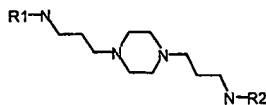
5 In a preferred embodiment, we describe a complex for inhibiting nucleic acid expression in a cell. The complex comprises mixing a siRNA and a compound or compounds to form the complex wherein the zeta potential of the complex is less negative than the zeta potential of the siRNA alone. Then inserting the complex into a mammalian blood vessel, *in vivo*, and delivering the complex to the cell wherein the expression of a selected gene is inhibited.

10 In a preferred embodiment, the polycation, the siRNA, or the amphipathic compound may be modified by attachment of a functional group. The functional group can be, but is not limited to, a targeting signal or a label or other group that facilitates delivery of the siRNA. The group may be attached to one or more of the components prior to complex formation. Alternatively, the group(s) may be attached to the complex after formation of the complex.

15 In a preferred embodiment the compound, compositions, and processes for delivery of a siRNA to an animal cell can be used wherein the cell is located *in vitro*, *ex vivo*, *in situ*, or *in vivo*.

20 In a preferred embodiment, the present invention describes a process of delivering a siRNA to an animal cell comprising associating the cell with a ternary complex comprising an amphipathic compound, an effective amount of a polycation and a selected siRNA in solution. The term deliver means that the siRNA becomes associated with the cell thereby altering the endogenous properties of the cell, by inhibiting expression of a gene. The
25 complex can be on the membrane of the cell or inside the cytoplasm, nucleus, or other organelle of the cell. Other terms sometimes used interchangeably with deliver include transfect, transfer, or transform.

In a preferred embodiment the present invention describes cationic amphipathic compounds, and the methods of preparation thereof, that enhance delivery of a siRNA to an animal cell
30 wherein the compounds have the general structure comprising:



structure #1

wherein R₁ and R₂ is selected from the group consisting of a C₆ to C₂₄ alkane, C₆-C₂₄ alkene, cycloalkyl, sterol, steroid, appropriately substituted lipid, acyl segment of a fatty acid, hydrophobic hormone, or other similar hydrophobic group.

5

The compound is considered cationic because the molecule has an overall positive charge (zeta potential that is positive). The compound is considered amphipathic because the molecule contains one end that is hydrophilic while the other end is hydrophobic.

- 10 Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Hydrocarbons are hydrophobic groups. Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and are hydrogen bond donors or acceptors with water. Examples of
- 15 hydrophilic groups include compounds with the following chemical moieties; carbohydrates, polyoxyethylene, peptides, oligonucleotides, and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls.

- In a preferred embodiment, these amphipathic compounds are combined with a polycation
- 20 and a siRNA to form a ternary complex which is then associated with an animal for the purpose of delivering the siRNA to the cell. In another preferred embodiment, these amphipathic compounds may also be combined with other amphipathic compounds, such as lipids to form liposomes which are then used to deliver a siRNA to an animal cell.

- 25 A siRNA is a nucleic acid that is a short, 15-50 base pairs and preferably 21-25 base pairs, double stranded ribonucleic acid. The term nucleic acid is a term of art that refers to a polymer containing at least two nucleotides. Natural nucleotides contain a deoxyribose (DNA) or ribose (RNA) group, a phosphate group, and a base. Bases include purines and pyrimidines, which further include the natural compounds adenine, thymine, guanine,
- 30 cytosine, uracil, inosine, and natural analogs. Synthetic derivatives of purines and pyrimidines include, but are not limited to, modifications which place new reactive groups on the base such as, but not limited to, amines, alcohols, thiols, carboxylates, and alkylhalides. The term base encompasses any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N⁶-methyladenosine, aziridinylcytosine,

- pseudoisocytosine, 5-(carboxyhydroxymethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. Nucleotides are the monomeric units of nucleic acid polymers and are linked together through the phosphate groups in natural polynucleotides. Natural polynucleotides have a ribose-phosphate backbone. Artificial or synthetic polynucleotides are polymerized *in vitro* and contain the same or similar bases but may contain a backbone of a type other than the natural ribose-phosphate backbone. These backbones include, but are not limited to: PNAs (peptide nucleic acids), phosphorothioates, phosphorodiamidates, morpholinos, and other variants of the phosphate backbone of natural polynucleotides.
- The siRNA contains sequence that is identical or nearly identical to a portion of a gene. RNA may be polymerized *in vitro*, recombinant RNA, contain chimeric sequences, or derivatives of these groups. The siRNA may contain ribonucleotides, deoxyribonucleotides, synthetic nucleotides, or any suitable combination such that expression of the target gene is inhibited. The RNA is preferably double stranded, but may be single, triple, or quadruple stranded.
- A delivered siRNA can stay within the cytoplasm or nucleus. The siRNA can be delivered to a cell to inhibit expression an endogenous or exogenous nucleotide sequence or to affect a specific physiological characteristic not naturally associated with the cell.
- Protein refers herein to a linear series of greater than 2 amino acid residues connected one to another as in a polypeptide. A "therapeutic" effect of the protein in attenuating or preventing the disease state can be accomplished by the protein either staying within the cell, remaining attached to the cell in the membrane, or being secreted and dissociated from the cell where it can enter the general circulation and blood. Secreted proteins that can be therapeutic include

hormones, cytokines, growth factors, clotting factors, anti-protease proteins (e.g., alpha-1-antitrypsin), angiogenic proteins (e.g., vascular endothelial growth factor, fibroblast growth factors), anti-angiogenic proteins (e.g., endostatin, angiostatin), and other proteins that are present in the blood. Proteins on the membrane can have a therapeutic effect by providing a
5 receptor for the cell to take up a protein or lipoprotein (e.g., low density lipoprotein receptor). Therapeutic proteins that stay within the cell ("intracellular proteins") can be enzymes that clear a circulating toxic metabolite as in phenylketonuria. They can also cause a cancer cell to be less proliferative or cancerous (e.g., less metastatic), or interfere with the replication of a virus. Intracellular proteins can be part of the cytoskeleton (e.g., actin, dystrophin, myosins,
10 sarcoglycans, dystroglycans) and thus have a therapeutic effect in cardiomyopathies and musculoskeletal diseases (e.g., Duchenne muscular dystrophy, limb-girdle disease). Other therapeutic proteins of particular interest to treating heart disease include polypeptides affecting cardiac contractility (e.g., calcium and sodium channels), inhibitors of restenosis (e.g., nitric oxide synthetase), angiogenic factors, and anti-angiogenic factors.

15 A siRNA can be delivered to a cell in order to produce a cellular change that is therapeutic. The delivery of siRNA or other genetic material for therapeutic purposes (the art of improving health in an animal including treatment or prevention of disease) is called gene therapy. The siRNA can be delivered either directly to the organism *in situ* or indirectly by
20 transfer to a cell *ex vivo* that is then transplanted into the organism. Entry into the cell is required for the siRNA to block the production of a protein or to decrease the amount of a RNA. Diseases, such as autosomal dominant muscular dystrophies, which are caused by dominant mutant genes, are examples of candidates for treatment with therapeutic siRNA. Delivery of siRNA would block production of the dominant protein thereby lessening the
25 disease.

A polycation means a polymer possessing net positive charge, for example poly-L-lysine hydrobromide, polyethylenimine, or histone. The polymeric polycation may contain
30 monomer units that are charge positive, charge neutral, or charge negative, however, the net charge of the polymer must be positive. A polycation also means a non-polymeric molecule that contains two or more positive charges.

A DNA-binding protein is a protein that associates with nucleic acid under conditions described in this application and forms a complex with nucleic acid with a high binding

constant. The DNA-binding protein can be used in an effective amount in its natural form or a modified form for this process. An "effective amount" of the polycation is an amount that will allow delivery of the siRNA to occur. In a preferred embodiment, the polycation is a polynucleotide-binding protein that is isolated from an animal tissue, such as calf thymus, or
5 produced in recombinant form from *E. coli*. Preferably, the polynucleotide-binding protein is cationic such as a histone protein. Histone H1 protein is the preferred histone type and can be purchased from several suppliers (Sigma, Invitrogen, etc).

Other Definitions:

10

Lipid

Any of a diverse group of organic compounds that are insoluble in water, but soluble in organic solvents such as chloroform and benzene. Lipids contain both hydrophobic and hydrophilic sections. Lipids is meant to include complex lipids, simple lipids, and synthetic
15 lipids.

Complex Lipids

Complex lipids are the esters of fatty acids and include glycerides (fats and oils), glycolipids, phospholipids, and waxes.

20

Simple Lipids

Simple lipids include steroids and terpenes.

Synthetic Lipids

25 Synthetic lipids includes amides prepared from fatty acids wherein the carboxylic acid has been converted to the amide, synthetic variants of complex lipids in which one or more oxygen atoms has been substituted by another heteroatom (such as Nitrogen or Sulfur), and derivatives of simple lipids in which additional hydrophilic groups have been chemically attached. Synthetic lipids may contain one or more labile groups.

30

Fats

Fats are glycerol esters of long-chain carboxylic acids. Hydrolysis of fats yields glycerol and a carboxylic acid – a fatty acid. Fatty acids may be saturated or unsaturated (contain one or more double bonds).

Oils

Oils are esters of carboxylic acids or are glycerides of fatty acids.

5 Glycolipids

Glycolipids are sugar containing lipids. The sugars are typically galactose, glucose or inositol.

Phospholipids

- 10 Phospholipids are lipids having both a phosphate group and one or more fatty acids (as esters of the fatty acid). The phosphate group may be bound to one or more additional organic groups.

Wax

- 15 Waxes are any of various solid or semisolid substances generally being esters of fatty acids.

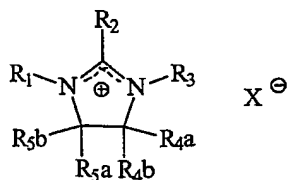
Fatty Acids

Fatty acids are considered the hydrolysis product of lipids (fats, waxes, and phosphoglycerides).

20

Polyimidazolinium:

- A polyimidazolinium is a polymer (random copolymer, block copolymer, or other copolymer) containing one or more imidazolinium subunits. A polyimidazolinium also means a homopolymer of the imidazolinium subunit. The imidazolinium subunit can be in the main chain of the polymer or as a side chain off of the polymer main chain. The polymer can be a
 25 net neutral polymer, a polycation, or a polyanion.

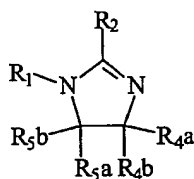
Imidazolinium (Imidazolinium Subunit):

In an imidazolinium (imidazolinium subunit), substituents R1, R2, R3, R4a, R4b, R5a, and R5b can independently be a hydrogen radical or a carbon radical with any substitution. The counterion (X) can be any counterion. Counterions include, but are not limited to chloride, bromide, iodide, and tosylate.

Poly-2-Imidazoline:

A poly-2-imidazoline is a polymer (random copolymer, block copolymer, or other copolymer) containing one or more imidazoline subunits. A poly-2-imidazoline also means a homopolymer of the 2-imidazoline subunit. The imidazoline subunit can be in the main chain of the polymer or as a side chain off of the polymer main chain. The polymer can be a net neutral polymer, a polycation, or a polyanion.

2-Imidazoline (2-Imidazoline Subunit):



In a 2-imidazoline (imidazoline subunit), substituents R1, R2, R4a, R4b, R5a, and R5b can independently be a hydrogen radical or a carbon radical with any substitution.

Complex

Two molecules are combined, to form a complex through a process called complexation or complex formation, if they are in contact with one another through noncovalent interactions such as electrostatic interactions, hydrogen bonding interactions, and hydrophobic interactions.

Modification

A molecule is modified, to form a modification through a process called modification, by a second molecule if the two become bonded through a covalent bond. That is, the two molecules form a covalent bond between an atom from one molecule and an atom from the second molecule resulting in the formation of a new single molecule. A chemical covalent bond is an interaction, bond, between two atoms in which there is a sharing of electron

density. Modification also means an interaction between two molecules through a noncovalent bond. For example crown ethers can form noncovalent bonds with certain amine groups.

5 Salt

A salt is any compound containing ionic bonds, that is bonds in which one or more electrons are transferred completely from one atom to another. Salts are ionic compounds that dissociate into cations and anions when dissolved in solution and thus increase the ionic strength of a solution.

10

Pharmaceutically Acceptable Salt

Pharmaceutically acceptable salt means both acid and base addition salts.

Pharmaceutically Acceptable Acid Addition Salt

15 A pharmaceutically acceptable acid addition salt is those salts which retain the biological effectiveness and properties of the free bases, and are not biologically or otherwise undesirable, and which are formed with inorganic acids such as hydrochloric acid, hydrobromic acid, sulfuric acid, nitric acid, phosphoric acid and the like, and organic acids such as acetic acid, propionic acid, pyruvic acid, maleic acid, malonic acid, succinic acid, 20 fumaric acid, tartaric acid, citric acid, benzoic acid, mandelic acid, methanesulfonic acid, ethansulfonic acid, p-toluenesulfonic acid, salicylic acid, trifluoroacetic acid, and the like.

Pharmaceutically Acceptable Base Addition Salt

25 A pharmaceutically acceptable base addition salt is those salts which retain the biological effectiveness and properties of the free acids, and are not biologically or otherwise undesirable. The salts are prepared from the addition of an inorganic base or an organic base to the free acid. Salts derived from inorganic bases include, but are not limited to, sodium, potassium, calcium, lithium, ammonium, magnesium, zinc, and aluminum salts and the like. Salts derived from organic bases include, but are not limited to salts of primary secondary, 30 and tertiary amines, such as methylamine, triethylamine, and the like.

Interpolyelectrolyte Complexes

An interpolyelectrolyte complex is a noncovalent interaction between polyelectrolytes of opposite charge.

Charge, Polarity, and Sign

The charge, polarity, or sign of a compound refers to whether or not a compound has lost one or more electrons (positive charge, polarity, or sign) or gained one or more electrons
5 (negative charge, polarity, or sign).

Cell Targeting Signals

Cell targeting signal (or abbreviated as the Signal) is defined in this specification as a molecule that modifies a biologically active compounds such as drug or nucleic acid and can
10 direct it to a cell location (such as tissue) or location in a cell (such as the nucleus) either in culture or in a whole organism. By modifying the cellular or tissue location of the foreign gene, the function of the biologically active compound can be enhanced.

The cell targeting signal can be a protein, peptide, lipid, steroid, sugar, carbohydrate, (non-
15 expressing) polynucleic acid or synthetic compound. The cell targeting signal enhances cellular binding to receptors, cytoplasmic transport to the nucleus and nuclear entry or release from endosomes or other intracellular vesicles.

Nuclear localizing signals enhance the targeting of the pharmaceutical into proximity of the
20 nucleus and/or its entry into the nucleus. Such nuclear transport signals can be a protein or a peptide such as the SV40 large T ag NLS or the nucleoplasmin NLS. These nuclear localizing signals interact with a variety of nuclear transport factors such as the NLS receptor (karyopherin alpha) which then interacts with karyopherin beta. The nuclear transport proteins themselves could also function as NLS's since they are targeted to the nuclear pore
25 and nucleus. For example, karyopherin beta itself could target the DNA to the nuclear pore complex. Several peptides have been derived from the SV40 T antigen. These include a short NLS or long NLS's and . Other NLS peptides have been derived from M9 protein, nucleoplasmin, and c-myc.

30 Signals that enhance release from intracellular compartments (releasing signals) can cause DNA release from intracellular compartments such as endosomes (early and late), lysosomes, phagosomes, vesicle, endoplasmic reticulum, golgi apparatus, trans golgi network (TGN), and sarcoplasmic reticulum. Release includes movement out of an intracellular compartment into cytoplasm or into an organelle such as the nucleus. Releasing signals include chemicals

such as chloroquine, bafilomycin or Brefeldin A1 and the ER-retaining signal (KDEL sequence), viral components such as influenza virus hemagglutinin subunit HA-2 peptides and other types of amphipathic peptides.

- 5 Cellular receptor signals are any signal that enhances the association of the biologically active compound with a cell. This can be accomplished by either increasing the binding of the compound to the cell surface and/or its association with an intracellular compartment, for example: ligands that enhance endocytosis by enhancing binding the cell surface. This includes agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or
10 galactose residues. Other proteins such as insulin, EGF, or transferrin can be used for targeting. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with thiol, sulfhydryl, or disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as lipids, fatty acids, cholesterol,
15 dansyl compounds, and amphotericin derivatives. In addition viral proteins could be used to bind cells.

Interaction Modifiers

- An interaction modifier changes the way that a molecule interacts with itself or other
20 molecules, relative to molecule containing no interaction modifier. The result of this modification is that self-interactions or interactions with other molecules are either increased or decreased. For example cell targeting signals are interaction modifiers with change the interaction between a molecule and a cell or cellular component. Polyethylene glycol is an interaction modifier that decreases interactions between molecules and themselves and with
25 other molecules.

Linkages

- An attachment that provides a covalent bond or spacer between two other groups (chemical moieties). The linkage may be electronically neutral, or may bear a positive or negative
30 charge. The chemical moieties can be hydrophilic or hydrophobic. Preferred spacer groups include, but are not limited to C1-C12 alkyl, C1-C12 alkenyl, C1-C12 alkynyl, C6-C18 aralkyl, C6-C18 aralkenyl, C6-C18 aralkynyl, ester, ether, ketone, alcohol, polyol, amide, amine, polyglycol, polyether, polyamine, thiol, thio ether, thioester, phosphorous containing, and heterocyclic. The linkage may or may not contain one or more labile bonds.

Bifunctional

Bifunctional molecules, commonly referred to as crosslinkers, are used to connect two molecules together, i.e. form a linkage between two molecules. Bifunctional molecules can
5 contain homo or heterobifunctionality.

Labile Bond

A labile bond is a covalent bond that is capable of being selectively broken. That is, the labile bond may be broken in the presence of other covalent bonds without the breakage of
10 other covalent bonds. For example, a disulfide bond is capable of being broken in the presence of thiols without cleavage of any other bonds, such as carbon-carbon, carbon-oxygen, carbon-sulfur, carbon-nitrogen bonds, which may also be present in the molecule. Labile also means cleavable.

Labile Linkage

A labile linkage is a chemical compound that contains a labile bond and provides a link or spacer between two other groups. The groups that are linked may be chosen from compounds such as biologically active compounds, membrane active compounds, compounds that inhibit membrane activity, functional reactive groups, monomers, and cell
20 targeting signals. The spacer group may contain chemical moieties chosen from a group that includes alkanes, alkenes, esters, ethers, glycerol, amide, saccharides, polysaccharides, and heteroatoms such as oxygen, sulfur, or nitrogen. The spacer may be electronically neutral, may bear a positive or negative charge, or may bear both positive and negative charges with an overall charge of neutral, positive or negative.

pH- Labile Linkages and Bonds

pH-labile refers to the selective breakage of a covalent bond under acidic conditions ($\text{pH} < 7$). That is, the pH-labile bond may be broken under acidic conditions in the presence of other covalent bonds without their breakage.

Amphiphilic and Amphipathic Compounds

Amphipathic, or amphiphilic, compounds have both hydrophilic (water-soluble) and hydrophobic (water-insoluble) parts. Hydrophilic groups indicate in qualitative terms that the chemical moiety is water-preferring. Typically, such chemical groups are water soluble, and

are hydrogen bond donors or acceptors with water. Examples of hydrophilic groups include compounds with the following chemical moieties; carbohydrates, polyoxyethylene, peptides, oligonucleotides and groups containing amines, amides, alkoxy amides, carboxylic acids, sulfurs, or hydroxyls. Hydrophobic groups indicate in qualitative terms that the chemical moiety is water-avoiding. Typically, such chemical groups are not water soluble, and tend not to hydrogen bonds. Hydrocarbons are hydrophobic groups.

Polymers

A polymer is a molecule built up by repetitive bonding together of smaller units called monomers. In this application the term polymer includes both oligomers which have two to about 80 monomers and polymers having more than 80 monomers. The polymer can be linear, branched network, star, comb, or ladder types of polymer. The polymer can be a homopolymer in which a single monomer is used or can be copolymer in which two or more monomers are used. Types of copolymers include alternating, random, block and graft. The main chain of a polymer is composed of the atoms whose bonds are required for propagation of polymer length. The side chain of a polymer is composed of the atoms whose bonds are not required for propagation of polymer length. To those skilled in the art of polymerization, there are several categories of polymerization processes that can be utilized in the described process. The polymerization can be chain or step. This classification description is more often used than the previous terminology of addition and condensation polymer. "Most step-reaction polymerizations are condensation processes and most chain-reaction polymerizations are addition processes" (M. P. Stevens Polymer Chemistry: An Introduction New York Oxford University Press 1990). Template polymerization can be used to form polymers from daughter polymers.

Step Polymerization:

In step polymerization, the polymerization occurs in a stepwise fashion. Polymer growth occurs by reaction between monomers, oligomers and polymers. No initiator is needed since there is the same reaction throughout and there is no termination step so that the end groups are still reactive. The polymerization rate decreases as the functional groups are consumed. Typically, step polymerization is done either of two different ways. One way, the monomer has both reactive functional groups (A and B) in the same molecule so that



Or the other approach is to have two difunctional monomers.



Generally, these reactions can involve acylation or alkylation. Acylation is defined as the introduction of an acyl group (-COR) onto a molecule. Alkylation is defined as the introduction of an alkyl group onto a molecule.

If functional group A is an amine then B can be (but not restricted to) an isothiocyanate, isocyanate, acyl azide, N-hydroxysuccinimide, sulfonyl chloride, aldehyde (including formaldehyde and glutaraldehyde), ketone, epoxide, carbonate, imidoester, carboxylate, or alkylphosphate, arylhalides (difluoro-dinitrobenzene), anhydrides or acid halides, p-nitrophenyl esters, o-nitrophenyl pentachlorophenyl esters, or pentafluorophenyl esters. In other terms when function A is an amine then function B can be acylating or alkylating agent or amination.

If functional group A is a thiol, sulfhydryl, then function B can be (but not restricted to) an iodoacetyl derivative, maleimide, aziridine derivative, acryloyl derivative, fluorobenzene derivatives, or disulfide derivative (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives).

If functional group A is carboxylate then function B can be (but not restricted to) a diazoacetate or an amine in which a carbodiimide is used. Other additives may be utilized such as carbonyldiimidazole, dimethylaminopyridine, N-hydroxysuccinimide or alcohol using carbodiimide and dimethylaminopyridine.

If functional group A is a hydroxyl then function B can be (but not restricted to) an epoxide, oxirane, or an amine in which carbonyldiimidazole or N, N'-disuccinimidyl carbonate, or N-hydroxysuccinimidyl chloroformate or other chloroformates are used.

If functional group A is an aldehyde or ketone then function B can be (but not restricted to) an hydrazine, hydrazide derivative, amine (to form a imine or iminium that may or may not be reduced by reducing agents such as NaCNBH₃) or hydroxyl compound to form a ketal or acetal.

Yet another approach is to have one difunctional monomer so that

A-A plus another agent yields -[A-A]-.

If function A is a thiol, sulfhydryl, group then it can be converted to disulfide bonds by oxidizing agents such as iodine (I₂) or NaIO₄ (sodium periodate), or oxygen (O₂). Function A can also be an amine that is converted to a thiol, sulfhydryl, group by reaction with 2-Iminothiolate (Traut's reagent) which then undergoes oxidation and disulfide formation. Disulfide derivatives (such as a pyridyl disulfide or 5-thio-2-nitrobenzoic acid{TNB} derivatives) can also be used to catalyze disulfide bond formation.

Functional group A or B in any of the above examples could also be a photoreactive group such as aryl azides, halogenated aryl azides, diazo, benzophenones, alkynes or diazine derivatives.

Reactions of the amine, hydroxyl, thiol, sulfhydryl, carboxylate groups yield chemical bonds that are described as amide, amidine, disulfide, ethers, esters, enamine, urea, isothiurea, isourea, sulfonamide, carbamate, carbon-nitrogen double bond (imine), alkylamine bond (secondary amine), carbon-nitrogen single bonds in which the carbon contains a hydroxyl group, thio-ether, diol, hydrazone, diazo, or sulfone.

Chain Polymerization: In chain-reaction polymerization growth of the polymer occurs by successive addition of monomer units to limited number of growing chains. The initiation and propagation mechanisms are different and there is usually a chain-terminating step. The polymerization rate remains constant until the monomer is depleted.

Monomers containing vinyl, acrylate, methacrylate, acrylamide, methacrylamide groups can undergo chain reaction, which can be radical, anionic, or cationic. Chain polymerization can also be accomplished by cycle or ring opening polymerization. Several different types of free radical initiations could be used that include peroxides, hydroxy peroxides, and azo compounds such as 2,2'-Azobis(-amidinopropane) dihydrochloride (AAP). A compound is a material made up of two or more elements.

Types of Monomers: A wide variety of monomers can be used in the polymerization processes. These include positive charged organic monomers such as amines, imidine, guanidine, imine, hydroxylamine, hydrazine, heterocycles (like imidazole, pyridine, morpholine, pyrimidine, or pyrene. The amines could be pH-sensitive in that the pK_a of the amine is within the physiologic range of 4 to 8. Specific amines include spermine, spermidine, N,N'-bis(2-aminoethyl)-1,3-propanediamine (AEPD), and 3,3'-Diamino-N,N'-dimethyldipropylammonium bromide.

Monomers can also be hydrophobic, hydrophilic or amphipathic. Monomers can also be intercalating agents such as acridine, thiazole orange, or ethidium bromide.

Other Components of the Monomers and Polymers: The polymers have other groups that increase their utility. These groups can be incorporated into monomers prior to polymer formation or attached to the polymer after its formation. These groups include: Targeting Groups- such groups are used for targeting the polymer-nucleic acid complexes to specific cells or tissues. Examples of such targeting agents include agents that target to the asialoglycoprotein receptor by using asialoglycoproteins or galactose residues. Other proteins

such as insulin, EGF, or transferrin can be used for targeting. Protein refers to a molecule made up of 2 or more amino acid residues connected one to another as in a polypeptide. The amino acids may be naturally occurring or synthetic. Peptides that include the RGD sequence can be used to target many cells. Chemical groups that react with thiol, sulfhydryl, or
5 disulfide groups on cells can also be used to target many types of cells. Folate and other vitamins can also be used for targeting. Other targeting groups include molecules that interact with membranes such as fatty acids, cholesterol, dansyl compounds, and amphotericin derivatives.

After interaction of the supramolecular complexes with the cell, other targeting groups can be
10 used to increase the delivery of the drug or nucleic acid to certain parts of the cell. For example, agents can be used to disrupt endosomes and a nuclear localizing signal (NLS) can be used to target the nucleus.

A variety of ligands have been used to target drugs and genes to cells and to specific cellular receptors. The ligand may seek a target within the cell membrane, on the cell
15 membrane or near a cell. Binding of ligands to receptors typically initiates endocytosis. Ligands could also be used for DNA delivery that bind to receptors that are not endocytosed. For example peptides containing RGD peptide sequence that bind integrin receptor could be used. In addition viral proteins could be used to bind the complex to cells. Lipids and steroids could be used to directly insert a complex into cellular membranes.

20 The polymers can also contain cleavable groups within themselves. When attached to the targeting group, cleavage leads to reduce interaction between the complex and the receptor for the targeting group. Cleavable groups include but are not restricted to disulfide bonds, diols, diazo bonds, ester bonds, sulfone bonds, acetals, ketals, enol ethers, enol esters, enamines and imines

25 **Polyelectrolyte**

A polyelectrolyte, or polyion, is a polymer possessing more than one charge, i.e. the polymer contains groups that have either gained or lost one or more electrons. A polycation is a polyelectrolyte possessing net positive charge, for example poly-L-lysine hydrobromide. The
30 polycation can contain monomer units that are charge positive, charge neutral, or charge negative, however, the net charge of the polymer must be positive. A polycation also can mean a non-polymeric molecule that contains two or more positive charges. A polyanion is a polyelectrolyte containing a net negative charge. The polyanion can contain monomer units that are charge negative, charge neutral, or charge positive, however, the net charge on the

polymer must be negative. A polyanion can also mean a non-polymeric molecule that contains two or more negative charges. The term polyelectrolyte includes polycation, polyanion, zwitterionic polymers, and neutral polymers. The term zwitterionic refers to the product (salt) of the reaction between an acidic group and a basic group that are part of the

5 same molecule.

Steric Stabilizer

A steric stabilizer is a long chain hydrophilic group that prevents aggregation of final polymer by sterically hindering particle to particle electrostatic interactions. Examples

10 include: alkyl groups, PEG chains, polysaccharides, alkyl amines. Electrostatic interactions are the non-covalent association of two or more substances due to attractive forces between positive and negative charges.

Buffers

15 Buffers are made from a weak acid or weak base and their salts. Buffer solutions resist changes in pH when additional acid or base is added to the solution.

Biological, Chemical, or Biochemical reactions

Biological, chemical, or biochemical reactions involve the formation or cleavage of ionic

20 and/or covalent bonds.

Reactive

A compound is reactive if it is capable of forming either an ionic or a covalent bond with another compound. The portions of reactive compounds that are capable of forming covalent

25 bonds are referred to as reactive functional groups.

Steroid

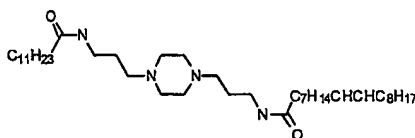
A steroid derivative means a sterol, a sterol in which the hydroxyl moiety has been modified (for example, acylated), or a steroid hormone, or an analog thereof. The modification can

30 include spacer groups, linkers, or reactive groups.

Sterics

Steric hindrance, or sterics, is the prevention or retardation of a chemical reaction because of neighboring groups on the same molecule.

Examples

5 1. Synthesis of Amphipathic Compounds:A. Synthesis of MC763:

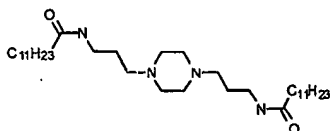
To a 25 mL flame dried flask was added oleoyl chloride (freshly distilled, 1.0 mL, 3.0 mmol, Aldrich Chemical Company) and lauroyl chloride (0.70 mL, 3.0 mmol, Aldrich Chemical Company) in 15 mL dichloromethane under N₂. The resulting solution was cooled to 0°C in an ice bath. N,N-Diisopropylethylamine (1.1 mL, 6.1 mmol, Aldrich Chemical Company) was added followed by 1,4-bis(3-aminopropyl)piperazine (0.50 mL, 2.4 mmol, Aldrich Chemical Company). The ice bath was removed and the solution stirred at ambient temperature for 15 hr. The solution was washed twice with 1N NaOH (10 mL), twice with water (10 mL), and concentrated under reduced pressure.

Approximately 30% of the resulting residue was purified by semi-preparative HPLC on a Beta Basic Cyano column (150 Å, 5 µm, 250x21 mm, Keystone Scientific, Inc.) with acetonitrile/H₂O/trifluoroacetic acid eluent. Three compounds were isolated from the column and verified by mass spectroscopy (Sciex API 150EX).

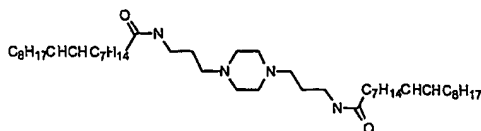
MC763 (MW= 647)

MC762 (MW= 564)

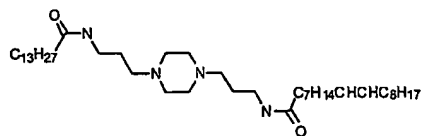
25 MC#798 (MW= 729.25)



MC762



MC#798

B. Synthesis of MC 765:

- 5 To a 25 mL flame dried flask was added oleoyl chloride (freshly distilled, 1.0 ml, 3.0 mmol) and myristoyl chloride (0.83 ml, 3.0 mmol, Aldrich Chemical Company) in 15 ml dichloromethane under N₂. The resulting solution was cooled to 0°C in an ice bath. N,N-Diisopropylethylamine (1.1 ml, 6.1 mmol) was added followed by 1,4-bis(3-aminopropyl)piperazine (0.50 ml, 2.4 mmol). The ice bath was removed and the solution
- 10 stirred at ambient temperature for 15 hr. The solution was washed twice with 1N NaOH (10 ml), twice with water (10 ml), and concentrated under reduced pressure.

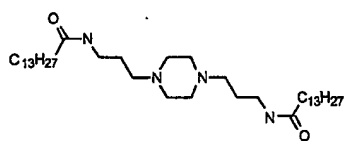
- Approximately 30% of the resulting residue was purified by semi-preparative HPLC on a Beta Basic Cyano column with acetonitrile/H₂O/trifluoroacetic acid eluent. Three compounds were
- 15 isolated from the column and verified by mass spectroscopy.

MC765 (MW= 674)

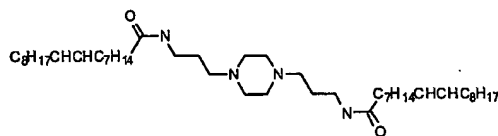
MC764 (MW= 620)

MC#798 (MW= 729.25)

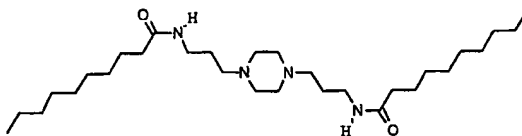
20



MC764



MC#798

C. Synthesis of MC774:

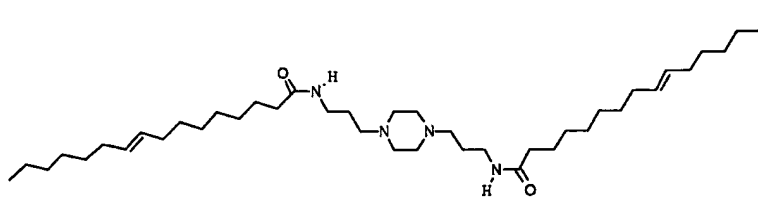
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To a solution of 1,4-bis(3-aminopropyl)piperazine (10 μ l, 0.049 mmol, Aldrich Chemical Company) in dichloromethane (1 ml) cooled to 0°C, was added decanoyl chloride (25 μ l, 0.12 mmol, Aldrich Chemical Company) and N,N-Diisopropylethylamine (21 μ l, 0.12 mmol). After 30 min, the solution was allowed to warm to ambient temperature. After 12 hrs, the solution was washed with water (2 \times 2 ml), and concentrated under reduced pressure to afford MC774 (21.6 mg, 87%) of sufficient purity by TLC.

D. Synthesis of MC775:

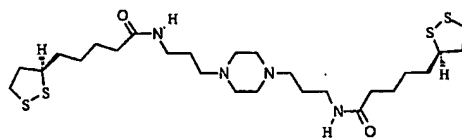
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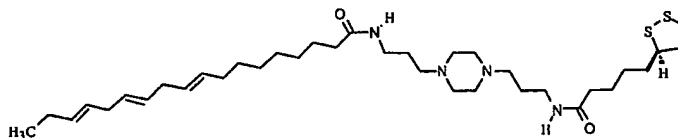
To a solution of 1,4-bis(3-aminopropyl)piperazine (10 μ l, 0.049 mmol) in dichloromethane (1 mL), was added palmitoleic acid (30.8 mg, 0.12 mmol, Aldrich Chemical Company), N,N-Diisopropylethylamine (21 μ l, 0.12 mmol), and dicyclohexylcarbodiimide (25 mg, 0.12 mmol). After 12 hrs, the solution was filtered and washed with water (2 \times 2 mL), and concentrated under reduced pressure to afford MC775 (26.5 mg, 81%) of sufficient purity by TLC.

E. Synthesis of MC777, MC778, and MC779:

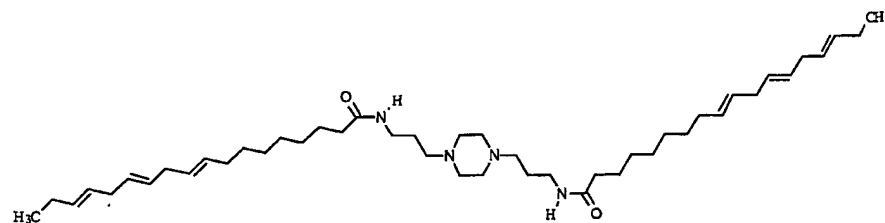
20



MC777



MC778

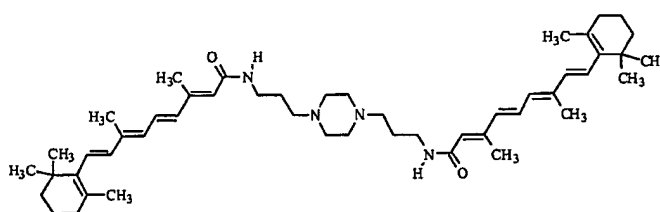


MC779

To a solution of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP, 1.300 g, 2.500 mmol, NovaBiochem) in dichloromethane (8 ml) was added thioctic acid (0.248 g, 1.20 mmol, Aldrich Chemical Company) and linolenic acid (365 μ l, 1.20 mmol, Aldrich Chemical Company). To the resulting solution was added 1,4-bis(3-aminopropyl)-piperazine (206 μ l, 1.00 mmol) followed by N,N-Diisopropylethylamine (610 μ l, 3.5 mmol). After 16 hrs at ambient temperature, the solution was washed with water (2 \times 20 ml), and concentrated under reduced pressure to afford 1.800 g of crude material. A 85 mg portion of the crude material was dissolved in 2 ml of acetonitrile (0.1% trifluoroacetic acid) / 1 ml of water (0.1% trifluoroacetic acid), and purified by reverse phase HPLC (10-90% B over 40 min) on a Beta Basic Cyano column to afford 31.8 mg MC777, 1.3 mg MC778, and 1.5 mg MC779.

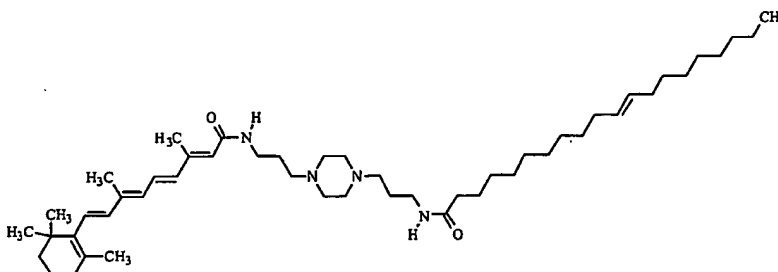
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F. Synthesis of MC780, MC781, and MC782:

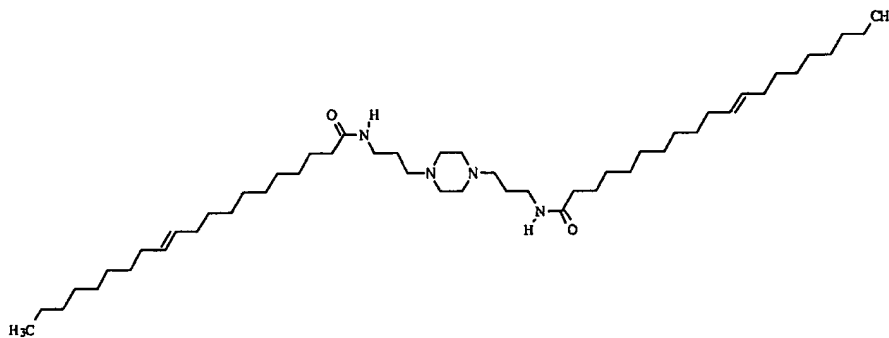


7.1 mg MC780

20



13.0 mg MC781



18.0 mg MC782

5

Compounds MC780, MC 781, and MC782 were made using a similar synthesis to compounds MC777, MC778, and MC779. The crude material from the synthesis was dissolved in 2 ml of acetonitrile (0.1% trifluoroacetic acid) / 1 ml of water (0.1% trifluoroacetic acid), and purified by reverse phase HPLC (10-90% B over 40 min) on a Beta Basic Cyano column to afford 7.1 mg MC780, 13.0 mg MC781, and 18.0 mg MC782.

10

G. Synthesis of Polysilazanes:

General experimental: The polyamine is dissolved in DMF to a concentration between 20 and 50 mg/mL. In a separate vessel, the chlorosilane is dissolved in THF to a concentration between 20 and 50 mg/mL. The appropriate amount of the chlorosilane solution (based on the molar ratio of amine residues to be modified) is added to the solution of the polyamine with mixing, resulting in the formation of a white solid. Water is added to the reaction vessel to a final concentration between 1 and 10 mg/mL based on the polyamine immediately prior to use. A solid support base may be included such as diisopropylaminomethyl polystyrene,

20

which is removed by filtration or centrifugation of the final solution. According to this general experimental procedure, the following compounds were prepared:

chlorosilane	polyamine (percent modification of amines -MC compound number)					
	PEI-800	PEI-1800	PEI-10k	brPEI-25k	IPEI-25k	E-PEI
$\begin{array}{c} \text{CH}_3 \\ \\ \text{Cl}-\text{Si}-\text{Cl} \\ \\ \text{CH}_3 \end{array}$	40-MC652 60-MC653	40-MC662 60-MC663 80-MC694	20-MC642 30-MC643	10-MC622 20-MC623 40-MC681	10-MC630 20-MC631	40-MC681
$\begin{array}{c} \text{tBu} \\ \\ \text{Cl}-\text{Si}-\text{Cl} \\ \\ \text{tBu} \end{array}$	40-MC654 60-MC655	40-MC664 60-MC665	20-MC644 30-MC645	10-MC624 20-MC625	10-MC632 20-MC633	
$\begin{array}{c} \text{Ph} \\ \\ \text{Cl}-\text{Si}-\text{Cl} \\ \\ \text{Ph} \end{array}$	40-MC656 60-MC657	40-MC666 60-MC667	20-MC646 30-MC647	10-MC626 20-MC627	10-MC634 20-MC635	
$\begin{array}{c} \text{H}_3\text{C} \quad \text{CH}_3 \\ \quad \\ \text{Cl}-\text{Si}-\text{CH}_2-\text{Si}-\text{Cl} \\ \quad \\ \text{H}_3\text{C} \quad \text{CH}_3 \end{array}$	10-MC616 20-MC617 30-MC660 60-MC661	10-MC618 20-MC619 40-MC670 60-MC671	20-MC650 30-MC651	10-MC607 20-MC608	10-MC609 20-MC610	
$\begin{array}{c} \text{iPr} \quad \text{iPr} \\ \quad \\ \text{Cl}-\text{Si}-\text{O}-\text{Si}-\text{Cl} \\ \quad \\ \text{iPr} \quad \text{iPr} \end{array}$	40-MC658 60-MC659	40-MC668 60-MC669	20-MC648 30-MC649	10-MC628 20-MC629	10-MC636 20-MC637	

5 Reagents:

brPEI-800, brPEI-1800; Polyethylenimine (base polymer average M_w ca. 800, 1800), Aldrich Chemical Company.

PEI-10k; Polyethylenimine (base polymer average M_w ca. 10,000), Polysciences, Inc.

brPEI-25k; branched Polyethylenimine (base polymer average M_w ca. 25000), Aldrich

10 Chemical Company.

IPEI-25k; linear-Polyethylenimine (base polymer average M_w ca. 25,000), Polysciences, Inc.

E-PEI; Polyethylenimine, 80-ethoxylated (base polymer average M_w ca. 50,000), Aldrich Chemical Company.

Dichlorodimethylsilane, Di-tert-butylchlorosilane, Dichlorodiphenylsilane, Aldrich

15 Chemical Company.

1,1,4,4-Tetramethyl-1,4-dichlorodisilylethylene, 1,3-Dichlorotetraisopropylidisiloxane, United Chemical Technologies, Inc.

Diisopropylaminomethyl polystyrene, Fluka Chemical Company.

2. Delivery of siRNA to Animal Cells *In Vitro*:

Use of Reporter Genes

A marker or reporter gene is a polynucleotide that encodes a gene product that can be easily
5 assayed, such as firefly luciferase or green fluorescent protein (GFP). The presence of the
product of the marker gene indicates that the cell is transfected and the amount of the product
indicates the efficiency of the transfection process. The luciferase reporter gene, in
conjunction with siRNA delivery methods, was used in our studies to quantitatively
determine the efficiency of siRNA delivery.

10

Preparation of Transfection Complexes:

The compositions, or ternary complexes, are prepared by mixing the polynucleotide with one
or more amphipathic compounds and an effective amount of a polycation. In one preferred
embodiment, the siRNA is mixed first with the polycation in serum-free media or other non-
15 toxic solution and the amphipathic compound is then added to the mixture. The mixture
containing the ternary complex of siRNA, polycation and amphipathic compound is then
added to the cells. In another preferred embodiment, the amphipathic compound is mixed
first with the polycation in solution and then the siRNA is added to the mixture. The mixture
containing the ternary complex of siRNA, polycation and amphipathic compound is then
20 added to the cells.

A. Delivery of siRNA to mammalian ATCC COS7 cells. COS7 cells were maintained in
Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum. All cultures
were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. Approximately 24
25 hours prior to transfection, cells were plated at an appropriate density in a T75 flask and
incubated overnight. At 50% confluency, cells were initially transfected with pGL-3-control
(firefly luciferase, Promega, Madison WI) and pRL-SV40 (sea pansy luciferase, Promega,
Madison, WI) using TransIT-LT1 transfection reagent according to the manufacturer's
recommendations (Mirus Corporation, Madison, WI). 15 µg pGL-3-control and 50 ng pRL-
30 SV40 were added to 45 µl TransIT-LT1 in 500 µl Opti-MEM (Invitrogen) and incubated 5
min at RT. DNA complexes were then added to cells in the T75 flask and incubated 2 h at
37°C. Cells were washed with PBS, harvested with trypsin/EDTA, suspended in media,
plated into a 24-well plate with 250 µl DMEM + 10% serum and incubated 2 h at 37°C.

siRNA-Luc⁺ (Dharmacon), 0.6 pmol, was then combined with the indicated delivery agent in 100 μ l Opti-MEM per well, incubated 5 min at RT and added to cells at 37°C.

5 The pGL-3-control plasmid contains the firefly luc⁺ coding region under transcriptional control of the simian virus 40 enhancer and early promoter region. The pRL-SV40 plasmid contains the coding region for *Renilla reniformis*, sea pansy, luciferase under transcriptional control of the Simion virus 40 enhancer and early promoter region.

10 Single-stranded, gene-specific sense and antisense RNA oligomers with overhanging 3' deoxynucleotides were prepared and purified by PAGE (Dharmacon, LaFayette, CO). The two complementary oligonucleotides, 40 μ M each, are annealed in 250 μ l 100mM NaCl /50mM Tris-HCl, pH 8.0 buffer by heating to 94°C for 2 minutes, cooling to 90°C for 1 minute, then cooling to 20°C at a rate of 1°C per minute. The resulting siRNA was stored at -20°C prior to use.

15 The sense oligonucleotide, with identity to the luc⁺ gene in pGL-3-control, had the sequence: 5'-rCrUrUrArCrGrCrUrGrArGrUrArCrUrUrCrGrATT-3', corresponding to positions155-173 of the luc⁺ reading frame. The letter "r" preceding a nucleotide indicates that the nucleotide is a ribonucleotide. The antisense oligonucleotide, with identity to the luc⁺ gene in pGL-3-control, had the sequence: 5'-rUrCrGrArArGrUrArCrUrCrArGrCrGrUrArArGTT-3' corresponding to positions173-155 of the luc⁺ reading frame in the antisense direction. The letter "r" preceding a nucleotide indicates that the nucleotide is a ribonucleotide. The annealed oligonucleotides containing luc⁺ coding sequence are referred to as siRNA-luc⁺.

25 Cells were harvested after 24 h and assayed for luciferase activity using the Promega Dual Luciferase Kit (Promega). A Lumat LB 9507 (EG&G Berthold, Bad-Wildbad, Germany) luminometer was used. The amount of luciferase expression was recorded in relative light units. Numbers were then adjusted for control sea pansy luciferase expression and are expressed as the percentage of firefly luciferase expression in the absence of siRNA.

30 Numbers are the average for at least two separate wells of cells.

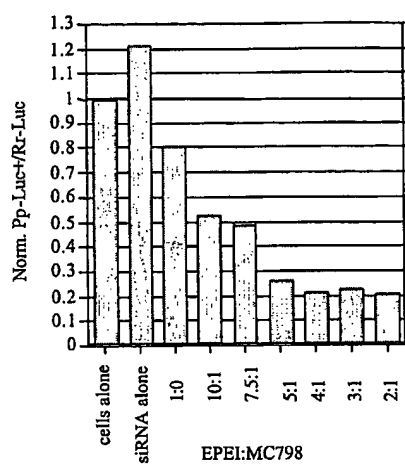


Table A. MC#798 addition to ePEI results in increased siRNA biological activity

- 5 The results in Table A show that addition of the amphipathic compound, MC#798, significantly enhances delivery of siRNA when combined with ePEI. Maximum delivery is achieved when the PEI/MC#798 ratio is 4:1 (wt/wt).

Table 1. Delivery of siRNA-Luc⁺ to pGL3-control transfected COS7 cells with ePEI:amphipathic compound formulations.

amphipathic compound		ratio	amount PEI + ?	siRNA nM	Luciferase Activity
none				0	1.000
ePEI	MC#798	4:1	6 µg	0	0.751
		4:1	6 µg	1	0.105
ePEI	MC762	3:1	6 µg	1	0.300
		4:1	6 µg	1	0.187
ePEI	MC763	3:1	6 µg	1	0.162
		4:1	6 µg	1	0.176
		4:1	8 µg	1	0.161
ePEI	MC762	3:1	6 µg	1	0.174

		4:1	6 μ g	1	0.278
		3:1	6 μ g	1	0.166
ePEI	MC765	4:1	6 μ g	1	0.126
		4:1	8 μ g	1	0.218
PEI			6 μ g	0	0.143
PEI			6 μ g	1	0.2903

The results demonstrate the effective delivery of siRNA to COS7 cells using the indicated compositions.

- 5 B. Ethoxylated-PEI/MC#798 mediated siRNA delivery is highly effective in inhibiting expression of target genes in mammalian cells in culture. We performed siRNA-Luc+ titrations in transiently transfected COS-7 cells to determine the IC₅₀ of siRNA-Luc+. Results indicate that the concentration of siRNA required to inhibit target Luc+ expression by 50% is approximately 0.18 nM (Table: B). This concentration is at least 100-fold less than that
- 10 required for even the most effective antisense molecules²⁹. Maximal inhibition by siRNA-Luc+ occurred between 25 and 100 nM and was nearly 95%. This is quite remarkable given the high level of expression afforded by the SV-40 enhancer contained in the plasmids used to drive luciferase expression. No inhibition was observed when either the sense or antisense RNA strand of siRNA-Luc+ (Table B) or when an siRNA targeted to sequences in the
- 15 plasmid backbone was delivered (data not shown). These results indicate that siRNA is a highly effective reagent for inhibiting specific gene expression.

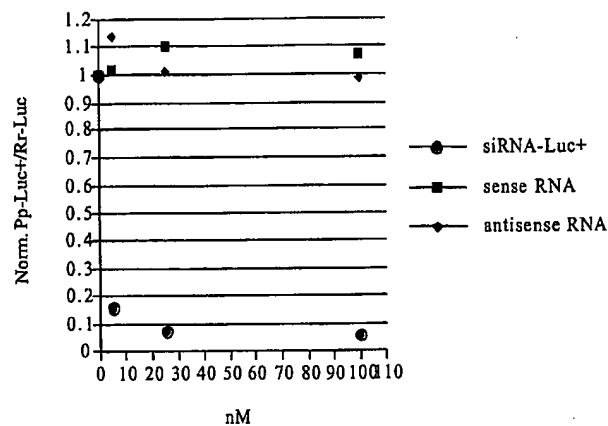


Table B: Delivery of siRNA-Luc+ using ePEI:MC#798 (4:1) results in strong and specific inhibition of Luc+ target gene expression in COS-7 cells in culture. The degree of inhibition of Luc+ by low concentrations of siRNA-Luc+ (0-1nM) indicated an IC_{50} of approximately 0.18 nM.

C. Ethoxylated-PEI/MC#798 is effective in delivering siRNA to multiple cell types. The effectiveness of siRNA was also tested in other mammalian cell lines transiently transfected with luciferase expression plasmids. Delivery of siRNA-Luc+ at 1 nM concentration to 293, HeLa, and CHO cells resulted in 70%, 94% and 87% inhibition, respectively, of the firefly luciferase target gene (Table: C). It is reasonable to expect that use of higher concentrations of siRNA would lead to even greater levels of inhibition. Experiments performed on mouse 3T3 cells stably transformed with a plasmid encoding the wild type version of firefly luciferase showed a 78% reduction in luciferase activity after delivery of 1 nM siRNA-Luc. Together these results indicate that siRNA is highly effective at inhibiting gene expression in both transiently and stably transformed mammalian cell lines.

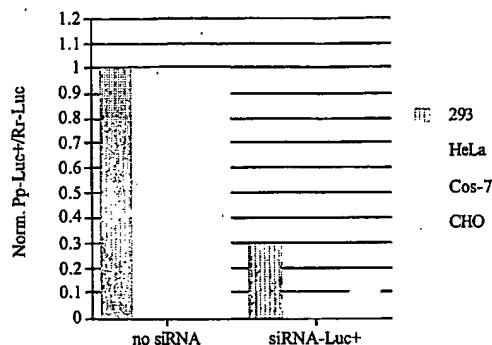


Table C: ePEI/MC#798 mediated delivery of siRNA results in strong inhibition of Luc+ expression in a variety of mammalian cell lines even at low siRNA concentration (1 nM).

5

D. Polysilazanes/MC798 are effective in delivering siRNA to COS7 cells:

By similar methods as described above MC681 has been shown to effectively deliver siRNA.

Table 2. Delivery of siRNA-Luc⁺ to pGL3-control transfected COS7 cells with ePEI:amphipathic compound formulations.

amphipathic compound		ratio	amount PEI + ?	siRNA nM	Luciferase Activity
none				0	1.000
MC681	MC798	3:1	6 µg	0	
		3:1	6 µg	1	
MC681	MC798	4:1	6 µg	0	
		4:1	6 µg	1	

10

E. Delivery of RNA oligo to HeLa-luc cells:

The delivery of RNA oligo for a positive readout was also conducted. A commercially-available HeLa cell line that carries an integrated luciferase gene with a mutant splice site was employed. This mutant splice site results in production of a mRNA coding for a truncated inactive luciferase protein. The blocking RNA base pairs to and thus blocks this

15

splice site, thereby enabling expression of the full-length active enzyme. Thus, the luciferase activity in this cell line is directly proportional to the amount of RNA delivered.

HeLa Luc/705 cells (Clontech Laboratories, Palo Alto, CA) were grown in as the standard HeLa cells. The cells were plated in 24-well culture dishes at a density of 3×10^6 cells/well and incubated for 24 hours. Media were replaced with 1.0 ml DMEM containing 2.5 nmol RNA oligo (2'OMe CCU CUU ACC UCA GUU ACA AUU UAU A, TriLink BioTechnologies, San Diego, CA) and polycation/amphipathic compound. The cells were incubated for 4 hours in a humidified, 5% CO₂ incubator at 37°C. The media was then replaced with DMEM containing 10% fetal bovine serum. The cells were then incubated for an additional 48 h. The cells were then harvested and the lysates were then assayed for luciferase expression as previously reported using a Lumat LB 9507 (EG&G Berthold, Bad-Wildbad, Germany) luminometer [Wolff, 1990 #72].

15 Results:

Table 3. Delivery of RNA-Oligo to HeLa-Luc cells with polycation:amphipathic compound formulations.

amphipathic compound		ratio	amount polycation	RNA nM	RLU
None				0	868
EPEI	MC798	3:1	5 µg	2.5	2594
		3:1	7.5 µg	2.5	5221
		3:1	10 µg	2.5	8141
		4:1	5 µg	2.5	5053
MC681		4:1	7.5 µg	2.5	27966
		4:1	10 µg	2.5	29582
			7.5 µg	2.5	12798
			10 µg	2.5	12533
			12.5 µg	2.5	8188
MC681	MC798	3:1	5 µg	2.5	10806
		3:1	7.5 µg	2.5	9709
		4:1	7.5 µg	2.5	25702

	4:1	10 μ g	2.5	14765
	4:1	12.5 μ g	2.5	9200

ePEI and MC681 with MC798 show delivery of the RNA oligo to HeLa-Luc cells.

F. siRNA mediated inhibition of a chromosomally integrated reporter gene.

5

Ideally, the inhibitory effect of siRNA on target gene expression would be complete and long-lasting and work well on endogenous genes. These characteristics would enable straightforward analysis of gene function without the complications that can arise from interpreting data after only partial or short-term inhibition. In the previous examples we

10 showed data that indicate that 95% inhibition can be achieved in cells lines transiently transfected with reporter genes. In order to determine if a chromosomally integrated gene (i.e. stably transfected) can be inhibited we performed an experiment using mouse NIH3T3 cells stably transfected with an expression plasmid encoding the wild type version of the firefly luciferase gene. EPEI + MC798 (TransIT-TKO) was used to deliver siRNA-Luc (or the

15 control siRNA-ori to cells in replicate wells) at a final concentration of 25 nM. Controls included cells receiving TransIT-TKO alone and untreated cells. The media was changed and the cells split every 4 days during the course of the experiment. Cells from replicate wells were harvested on days 1, 2, 3, 7, 10 and 14 after transfection and assayed for Luciferase activity. Results indicate that siRNA-Luc inhibition of Luciferase expression was in the 80-

20 95% range on each day assayed (see Table B below). The levels of Luciferase activity in cells transfected with control siRNA or treated with TransIT-TKO alone were not significantly different than those in untreated cells. These results indicate that siRNA-mediated RNAi is highly effective and long lasting in these cultured cells, a result that is consistent with RNAi observed in studies performed on mouse oocytes where RNAi was observed over a 50 to 100-

25 fold increase in cells mass 50, 51.

30

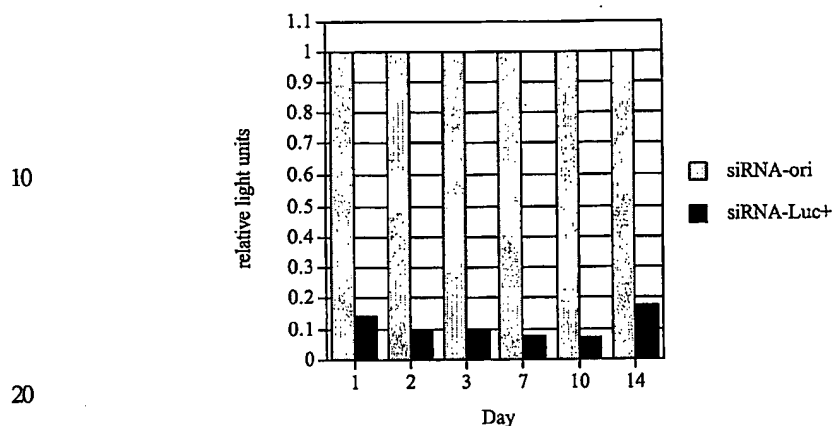


Table B: Single application of TransIT-TKO delivered siRNA-Luc results in long-term inhibition of Luciferase expression. The data is normalized to cells receiving the control, siRNA-ori.

G. siRNA mediated inhibition of endogenous gene expression (nuclear lamin A/C) using EPEI and MC798.

EPEI + MC798 (TransIT-TKO) was used to deliver siRNA-Luc [or the control siRNA-ori to CHO (chinese hamster ovary) cells in replicate wells] at a final concentration of 25 nM. Controls included cells receiving TransIT-TKO alone and untreated cells. The media was changed and the cells split every 4 days during the course of the experiment. Cells from replicate wells were harvested 2 days after transfection and total cellular protein was purified. Aliquots of protein from cells transfected with siRNA targeted for lamin A/C (and control siRNA) were run on a SDS-PAGE (3-12% gradient gel) and electrotransferred to nylon membranes. Protein expression of nuclear lamin A/C was quantitated via western blot analysis (anti-mouse lamin A/C) Results indicate that siRNA-lamin A/C inhibition of nuclear lamin expression was in the 80-95% range on the day assayed. The levels of lamin A/C in cells transfected with control siRNA or treated with TransIT-TKO alone were not significantly different than those in untreated cells. These results indicate that siRNA-mediated RNAi is highly effective in inhibiting expression of an endogenous cellular gene.

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The foregoing is considered as illustrative only of the principles of the invention.

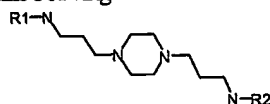
Furthermore, since numerous modifications and changes will readily occur to those skilled in the art, it is not desired to limit the invention to the exact construction and operation shown and described. Therefore, all suitable modifications and equivalents fall within the scope of

5 the invention.

10

We claim:

1. A deliverable composition comprising: an amphipathic compound, a polycation, and a siRNA.
2. The composition of claim 1 wherein the polycation is a DNA-binding protein.
3. The composition of claim 2 wherein the DNA-binding protein is a histone.
4. The composition of claim 3 wherein the histone is histone H1.
5. The composition of claim 1 wherein the polycation is a polymer.
6. The amphipathic compound of claim 1 having the structure comprising:



wherein R1 and R2 are selected from the group consisting of a C6 to C24 alkane, C6-C24 alkene, cycloalkyl, sterol, steroid, substituted lipid, acyl segment of a fatty acid, hydrophobic hormone, and hydrophobic hormone analog.

7. The amphipathic compound of claim 6 wherein R1 and R2 are the same.
8. The amphipathic compound of claim 6 wherein R1 and R2 are different.
9. A process of delivering a siRNA to an animal cell comprising: associating the cell with a composition comprising an amphipathic compound, an effective amount of a polycation, and a siRNA in solution.
10. The process of claim 9 wherein the polycation is a DNA-binding protein.
11. The process of claim 10 wherein the DNA-binding protein is a histone.
12. The process of claim 11 wherein the histone is histone H1.
13. The process of claim 9 wherein the polycation is a polymer.
14. The process of claim 9 wherein the animal cell is *in vivo*.
15. A process of claim 9 wherein the animal cell is *in vitro*.
16. The process of claim 9 wherein the animal cell is *ex vivo*.
17. The process of claim 9 wherein the animal cell is a mammalian cell.

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/17556

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C12N 15/64; A61K 38/16; C07H 21/00

US CL : 435/172.3; 530/358; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : asdf

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,777,153 A (LIN et al.) 09 July 1998 (07.07.1998) Col. 1, line 60, col. 7, line 65, col. 9, lines 23-28, line 68+, col. 10, line 35+, claims 1, 4, and 20.	1, 5, 6, 8, 9, 13-17 ----- 1-17
X --- Y	US 5,744,335 A (WOLFF et al) 28 April 1998 (28.04.1998) See entire document, particularly the abstract, structures at col. 1-3, col. 8 line 25+, col. 7 line 25+, claims 1-8	1-17 ----- 1-17

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.*** Special categories of cited documents:****"A"** document defining the general state of the art which is not considered to be of particular relevance**"B"** earlier application or patent published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art**"&"** document member of the same patent family

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23 August 2002 (23.08.2002)

Date of mailing of the international search report

04 FEB 2003

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